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# Multi-photon fluorescence spectroscopy of fluorescent bio-probes and bio-molecules

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## ABSTRACT

Multi-photon fluorescence spectra of a number of commonly used biological probes were measured in this study. Significant spectral variation has been detected between single and multi-photon excitation. The result is important for the proper selection of spectral setting/ dichroic beam splitter in the set-up of a multi-photon fluorescence microscope. The information can also be useful in the detection of multi-photon fluorescence in bio-chip technology. In addition, we have investigated a few highly fluorescent bio-molecules commonly found in plant cells.

**Keywords:** Multi-photon fluorescence spectroscopy, two-photon, three-photon, fluorescence probes, biological probes, auto-fluorescence, bio-chip

## 1. INTRODUCTION

Fluorescent probes are commonly used in biological fluorescence microscopy for tracking specific structures and subcellular compartments, for measuring cellular ionic conditions and for indication of cell survival. For instance, FITC is routinely used to tag antibodies to localize specific antigen; Indo-1 is used to measure the  $\text{Ca}^{++}$  concentration within a cell and Calcein is used as an indicator for the integrity of the cell membrane. Recent development in multi-photon fluorescence microscopy has greatly expanded the usage of these fluorescent probes in biomedical research. Considering its non-linear nature, two-photon excitation may generate very different fluorescence spectral response in the sample when compared with single photon excitation<sup>1-4</sup>. It is thus necessary to measure the two-photon spectra of various fluorescent probes, so that two-photon fluorescence microscopy may be performed effectively and the images properly interpreted. Knowledge on the fluorescent properties of these dyes is also required for bio-chip technology. This report represents the third installment of a continued effort in characterizing the multi-photon fluorescence spectra of some commonly used bio-probes<sup>5,6</sup>. In addition, the auto-fluorescence in biological specimen frequently contributes to background noise in fluorescence microscopy and in bio-chip signal detection, thus characterization of the two-photon fluorescence properties of bio-molecules is necessary.

## 2. MATERIALS AND METHODS

### 1. Optical set-up

Two-photon fluorescence spectra excited with near infrared at 780nm were obtained with a SpectraPro-500 spectrophotometer (Acton Research) equipped with a TE-cooled PMT and coupled to a Spectra-Physics Tsunami Ti:sapphire laser pumped by a Coherent Verdi solid-state laser operated at 82MHz, 100fs pulse. The 1240nm infrared (IR) excitation was obtained from a Spectra-Physics Millennia IR (1064nm) pumped Chromium-doped Forsterite laser (built by CKS)<sup>7</sup> operated at 120MHz, 130fs pulse. A cooled CCD array spectrophotometer (Acton Research) was used for spectral detection. An

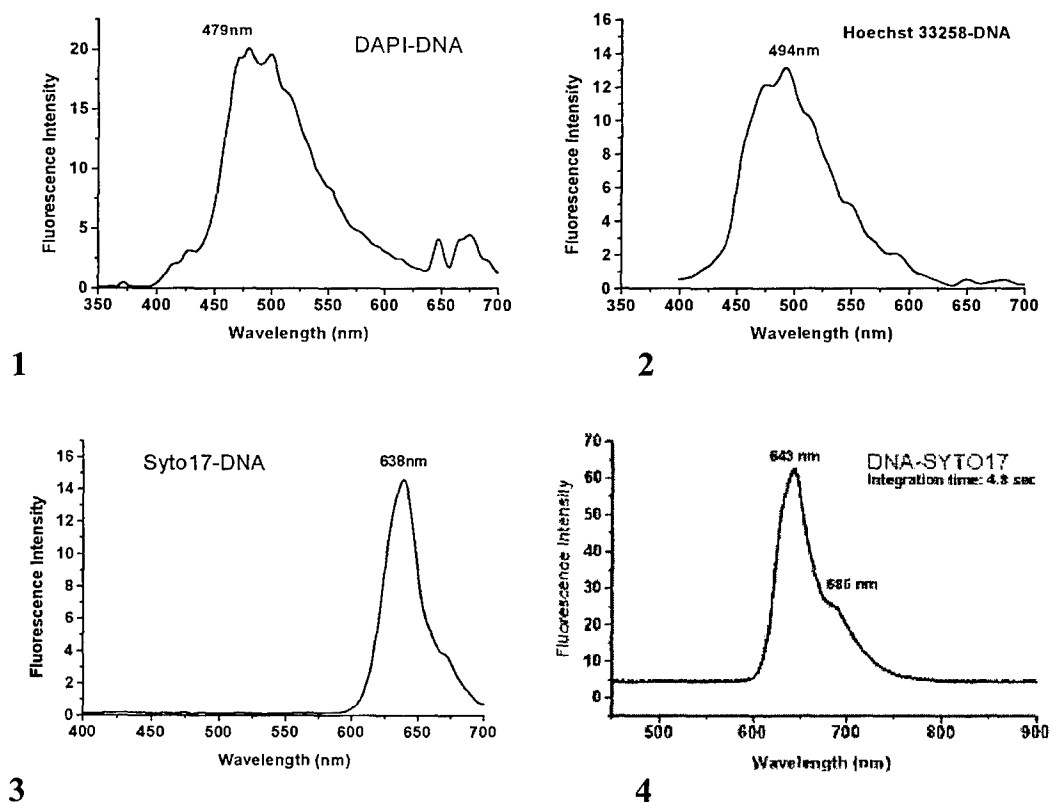
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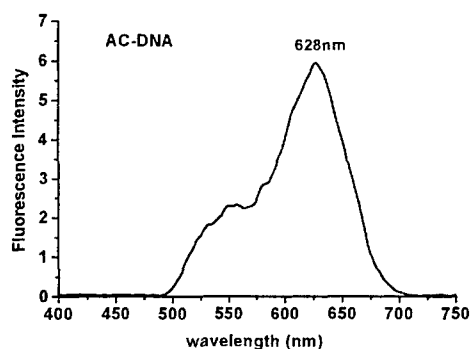
Olympus BX microscope trinocular head and an epi-fluorescence beam-splitter housing were modified for the measurements. A 740nm dichroic beam splitter was used for separating the excitation beam and the fluorescence emission. In addition, two IR cut-off filters (Edmond Scientific, Cat. K53-710) were installed in front of the entrance slit of the monochromator to reject scattered IR from the sample. A 4x microscope objective was used to focus the pump beam into a 0.3ml microfuge tube. For DNA probes, 2 $\mu$ M dye in the presence of 160 $\mu$ g/ml fragmented salmon sperm DNA in TE buffer (10mM Tris, 1mM EDTA, pH7.4)Fluorescence samples were prepared for spectral analysis. Methanol was used as solvent for MitoTracker and LysoTracker. Aqueous solutions of riboflavin, NADH and NADPH and acetone solution of chlorophyll a and chlorophyll b were used in this study.

### 3. RESULT AND DISCUSSION

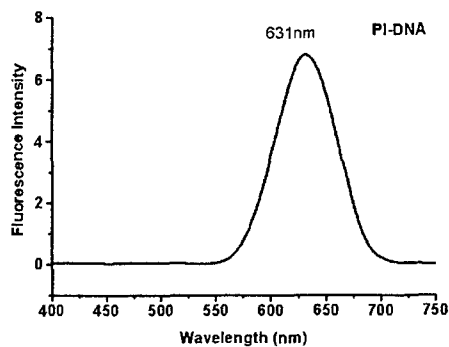
Figures 1- 12 show two-photon pumped fluorescence spectra of seven commonly used DNA-probes. Measurements of DAPI (Fig. 1), Hoechst 33258 (Fig. 2), Syto 17 [Fig. 3 (Ex=780nm) and Fig. 4 (Ex=1240nm)], Acridine Orange (Figure 5), Propidium Iodide (Figure 6), and Ethidium bromide (Figure 7) were performed with 2 $\mu$ M dye in the presence of 160 $\mu$ g/ml fragmented salmon sperm DNA in TE buffer (10mM Tris, 1mM EDTA, pH7.4). This concentration approximates 50 base pairs of DNA per dye molecule. In addition, methanol solutions of MitoTracker® (Molecular Probe M-7512; Fig. 8 and LysoTracker Red® (Molecular Probe L-7528; Fig. 9) were used in the measurement. Spectra in Figures 8 and 9 were excited with IR at 1240nm, therefore, the MitoTracker emission is the result of three-photon excitation, while the spectrum of LysoTracker is a mixed result of two and three-photon excitation. Table1 summarize the emission maximum of the above mentioned dyes and compare with single-photon emission maximum published by Molecular Probes Inc. Figure 10-12 show two-photon excited fluorescence spectra of riboflavin (Fig. 10), NADH (Fig. 11) and NADPH (Fig. 12). Table 2 summarize the emission maximum of riboflavin, NADH, NADPH, chlorophyll a and chlorophyll b.



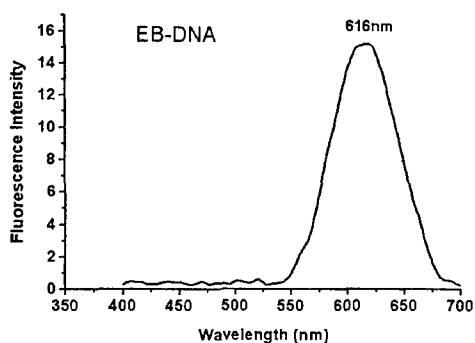
Figures 1-4. Two-photon pumped fluorescence spectra of DAPI [Figure 1 (Ex=780nm)], Hoechst 33258 [Figure 2 (Ex=780nm)], SYTO 17 [Figure 3 (Ex=780nm), Figure 4 (Ex=1240nm)].



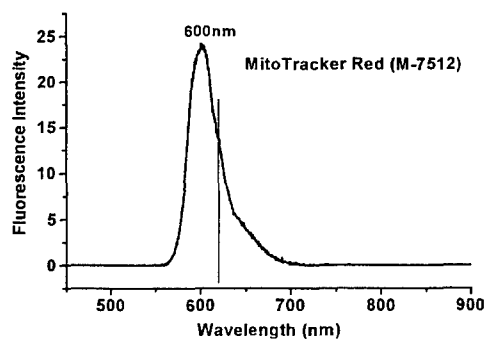
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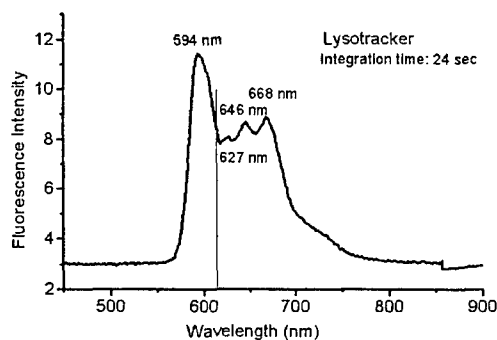
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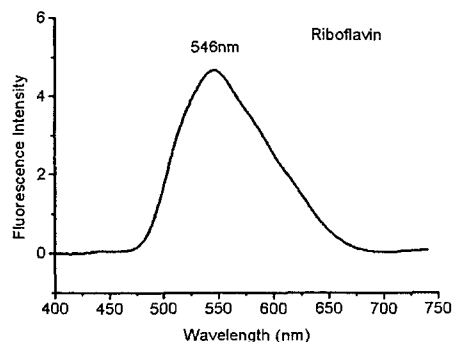
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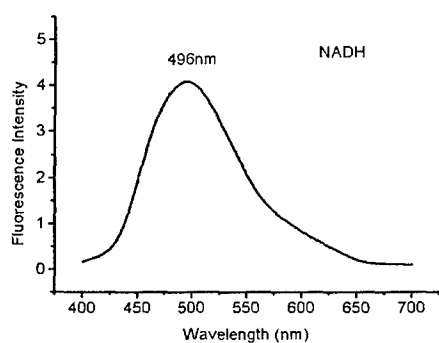


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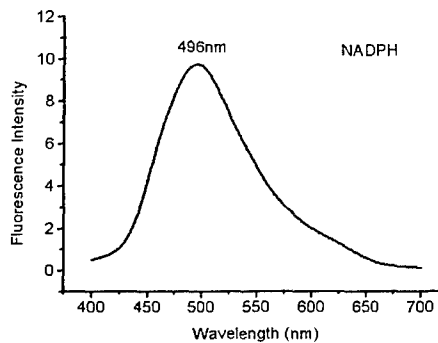


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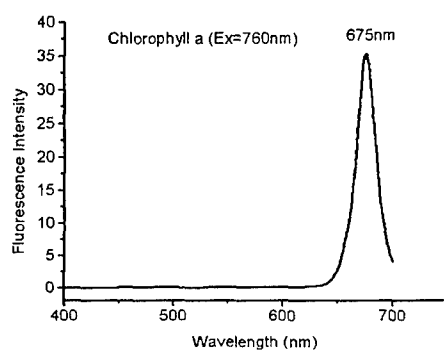
Figure 5-10. Acridine orange [Figure 5 (Ex=780nm)], Propidium Iodide [Figure 6 (Ex=780nm)], ethidium bromide [Figure 7 (Ex=780nm)], Mitotracker®M-7512 [Figure 8 (Ex=1240nm)], Lysotracker®L-7528 [Figure 9 (Ex=1240nm)]. Riboflavin [Figure 10 (Ex=780nm)]. The vertical line in Figure 8 and 9 indicates the  $1/2\lambda$  of the excitation wavelength.



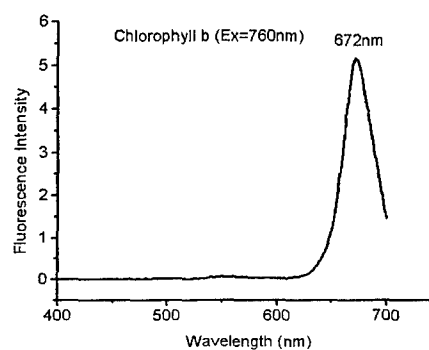
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Figure 11-14. Two-photon excited fluorescence spectra of NADH [Figure 11 (Ex=780nm)], NADPH [Figure 12 (Ex=780nm)], chlorophyll a [Figure 13 (Ex=760nm)] and chlorophyll b [Figure 14 (Ex=760nm)].

	1P (Em)*	2P Em (Ex=780nm)	2P Em (Ex=1234nm)
DAPI - DNA	461nm	479nm	-
Hoechst 33258 - DNA	461nm	494nm	-
Syto 17 -DNA	640nm	638nm	643nm
Acridine orange -DNA	626nm	628nm	-
Propidium Iodide -DNA	617nm	631nm	-
Ethidium bromide - DNA	605nm	616nm	-
Mitotracker®Red M-7512	599nm	-	600nm (2P and 3P)
Lysotracker®Red L-7528	592nm	-	594nm (3P), 646nm (2P), 668nm (2P)

Table 1. Single-photon (1P) and two-photon (2P) excited fluorescence emission maximum of various DNA dyes. 3P: three-photon excited fluorescence. Ex: excitation wavelength, Em: emission maximum. \*Data adopted from Molecular Probes Catalog (1999).

	2P
Riboflavin	546nm (Ex=780nm)
NADH	496nm (Ex=780nm)
NADPH	496nm (Ex=780nm)
Chlorophyll a	675nm (Ex=760nm)
Chlorophyll b	672nm (Ex=760nm)

Table 2. Two-photon (2P) excited fluorescence emission maximum of five bio-molecules. Ex: excitation wavelength.

### ACKNOWLEDGEMENTS

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